PROCESS FOR THE MANUFACTURE OF CAROTENOIDS AND BIOLOGICALLY USEFUL MATERIALS THEREOF

FIELD OF THE INVENTION

The present invention relates to the recombinant production of carotenoids, particularly astaxanthin, and to biologically useful materials therefor.

BACKGROUND OF THE INVENTION

Astaxanthin is known to be distributed in a wide variety of organisms, such as animals (e.g. birds such as flamingo and scarlet ibis, and fish such as rainbow trout and salmon), algae and microorganisms. It is also recognized that astaxanthin has a strong antioxidation property against active oxygen as well as most carotenoids. This property is expected to apply to the pharmaceutical usage of astaxanthin to protect living cells against some diseases, such as a cancer. Moreover, from the industrial application viewpoint, a demand for astaxanthin as a coloring reagent is increasing, especially in the farmed fish industry, such as salmon, because astaxanthin imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the marketplace.

Phaffia rhodozyma is known as a carotenogenic yeast strain that produces astaxanthin specifically. Different from the other carotenogenic yeast, *Rhodotorula*, *Phaffia rhodozyma* can ferment some sugars such as D-glucose. This is an important feature from an industrial application viewpoint. In a recent taxonomic study, a sexual cycle of *P. rhodozyma* was revealed and its telemorphic state was designated under the name of *Xanthophyllomyces dendrorhous* (W.I. Golubev; *Yeast* 11, 101 - 110, 1995). Some strain improvement studies to obtain hyper-producers of astaxanthin from *P. rhodozyma* have been conducted, however, in this decade, such effort have been restricted to employing the method of conventional mutagenesis and protoplast fusion. Recently, Wery *et al.* developed a host vector system using *P. rhodozyma* in which a non-replicable plasmid was used for multicopy integration onto the genome of *P. rhodozyma* at the ribosomal DNA locus (Wery *et al., Gene*, 184, 89-97, 1997). Verdoes *et al.* reported the use of improved vectors to obtain a transformant of *P. rhodozyma*, as well as its three carotenogenic genes which code for the enzymes that catalyze the reactions from geranylgeranyl pyrophosphate to beta-carotene (International patent WO97/23633). The

importance of genetic engineering methods to *P. rhodozyma* strain improvement studies should increase in the near future in order to surpass the productivity levels reached by conventional methods.

As described above, astaxanthin has an antioxidant property. This feature seems to have an important role *in vivo* for a protecting against active oxygen species such as O_2•, H_2O_2 and OH•, which are continuously generated in living cells. An *et al.* obtained a hyperproducer of astaxanthin from *P. rhodozyma* by selecting for an antimycin-sensitive strain after conventional chemical mutagenesis (An, G-H. *et al.*, *Appl. Env. Microbiol.*, 55 (1), 116-124, 1989). Antimycin is known to be an inhibitor of respiratory chain between cytochrome b and C_1 (Lucchini, G. *et al.*, *Mol. Gen. Genet.*, 177, 139- , 1979) and such antimycin-sensitive mutants displayed enhanced pigmentation. Furthermore, active oxygen species produced due to a blockade of the primary respiratory chain at the bc_1 complex stimulated carotenoid formation (An, G-H *et al.*, *Appl. Env. Microbiol.*, 55, 116-124, 1989). Indeed, addition of an O_2• generator, duroquinone, to the growth medium increased total carotenoid content (the main carotenoid is astaxanthin) as well as the relative amounts of xanthophylls present in *P. rhodozyma*, while the active oxygen species-quenching factor mannitol reversed this effect (Schroeder, W. A. *et al.*, *J. Gen. Microbiol.*, 139, 907-912, 1993). These results prompted the authors to speculate on the antioxidant property of astaxanthin in *P. rhodozyma*. In fact, astaxanthin production is stimulated in post-exponential growth phase when respiration activity is fully induced. Moreover, the addition of respiratory substrate, such as ethanol, to the medium enhanced astaxanthin production in *P. rhodozyma* (Gu, W-L. *et al.*, *J. Ind. Microbiol. Biotechnol.*, 19, 114-117, 1997). Schroeder *et al.* tried to determine the relationship of the superoxide dismutase (SOD) and catalase activities, which act as native active oxygen species-quenching factors in *P. rhodozyma* to the productivity of astaxanthin, by comparing the difference between a parent strain and an antimycin-sensitive hyperproducer of astaxanthin. However, direct correlation of *in vitro* activity could not be observed.

SUMMARY OF THE INVENTION

In accordance with the present invention, the genes and the enzymes for active oxygen species-quenching factor(s), such as SOD and catalase are provided. SOD and catalase are biological materials that improve the carotenoid production process. This invention involves the cloning and the determination of the genes that code for mitochondrial and cytoplasmic SODs and catalase. This invention also involves enzymatically characterizing the result of disrupting these genes in *P. rhodozyma*. The

disruption effects on the carotenogenesis can be confirmed by the cultivation of such transformants in an appropriate medium under an appropriate cultivation conditions.

More particularly, the present invention provides a process for producing carotenoids, which comprises cultivating a recombinant organism, whose gene for one or more active oxygen species-quenching factor(s) is substantially disrupted with the aid of a disruption cassette specific to the gene, and recovering carotenoids from the culture. The host organism of the recombinant organism may belong to the kingdom of *Monera, Protista* or *Fungi*. More preferably, the host organism of the recombinant organism may belong to the genus *Erwinia, Rhodobacter, Myxococcus, Flavobacter, Paracoccus, Synechococcus, Synechocystis, Agrobacterium, Streptomyces, Haematococcus, Dunaliella, Phaffia, Xanthophyllomyces, Neurospora, Rhodotorula, Blakeslea,* or *Phycomyces*. Most preferably, the host organism is a strain of *P. rhodozyma*

The active oxygen species-quenching factor(s) is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD), and/or catalase.

The present invention also provides a recombinant organism capable of producing carotenoids, characterized by the gene for at least one active oxygen species-quenching factor, that is substantially disrupted by introducing a disruption cassette specific to the gene. The active oxygen species-quenching factor(s) to be disrupted is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.

The present invention further provides a disruption cassette that is used to prepare the recombinant organism of the present invention by disrupting a gene coding for an active oxygen species-quenching factor effective in an organism that produces carotenoids. The disruption cassette includes a partial nucleotide sequence substantially identical to a part of the DNA sequence coding for an active oxygen species-quenching factor, and a selectable marker gene. For the construction of the disruption cassette, the target organism may belong to the kingdom of *Monera, Protista* or *Fungi*, more preferably to the genus *Erwinia, Rhodobacter, Myxococcus, Flavobacter, Paracoccus, Synechococcus, Synechocystis, Agrobacterium, Streptomyces, Haematococcus, Dunaliella, Phaffia, Xanthophyllomyces, Neurospora, Rhodotorula, Blakeslea,* or *Phycomyces*. The active oxygen species-quenching factor(s) to be disrupted is(are) mitochondrial superoxide dismutase (SOD), cytoplasmic superoxide dismutase (SOD) and/or catalase.

As used herein, a polynucleotide or polypeptide sequence (A) is said to be substantially identical to another sequence (B) if sequence A is at least 75% identical, preferably 85% identical, such as at least 95% identical to sequence B.

Further, the present invention provides a recombinant DNA sequence coding for an active oxygen species-quenching factor effective in an organism capable of producing carotenoids. The DNA sequence is isolated from an organism belonging to the kingdom of *Monera, Protista* or *Fungi*, more preferably to the genus *Erwinia, Rhodobacter, Myxococcus, Flavobacter, Paracoccus, Synechococcus, Synechocystis, Agrobacterium, Streptomyces, Haematococcus, Dunaliella, Phaffia, Xanthophyllomyces, Neurospora, Rhodotorula, Blakeslea,* or *Phycomyces*. Particularly preferred organism is *P. rhodozyma*. The active oxygen species-quenching factor coded by the recombinant DNA sequence may be mitochondrial superoxide dismutase, cytoplasmic superoxide dismutase and/or catalase.

A recombinant DNA sequence coding for mitochondrial superoxide dismutase is identified by SEQ ID NO: 1 or 4, or it may have sufficiently high homology to the sequence of SEQ ID NO: 1 or 4 to hybridize under high stringency hybridization and wash conditions with either of the sequences of SEQ ID NOs: 1 and 4. A recombinant DNA sequence coding for cytoplasmic superoxide dismutase is identified by SEQ ID NO: 2 or 6, or it may have sufficiently high homology to the sequence of SEQ ID NO: 2 or 6 to hybridize under high stringency hybridization and wash conditions with either of the sequences of SEQ ID NOs: 2 and 6. Also a recombinant DNA sequence coding for catalase is identified by SEQ ID NO: 3 or 8, or it may have sufficiently high homology to the sequence of SEQ ID NO: 3 or 8 to hybridize under high stringency hybridization and wash conditions with either of the sequences of SEQ ID NOs: 3 and 8.

In the present invention, any combination of the following hybridization and wash conditions may be used, as appropriate, to identify homologous polynucleotide sequences:

High Stringency Hybridization:

6X SSC

0.5% SDS

100 ug/ml denatured salmon sperm DNA

50% formamide

Incubate overnight with gentle rocking at 42°C overnight.

High Stringency Wash:

1 wash in 2X SSC, 0.5% SDS at Room Temperature for 15 minutes,

followed by another wash in 0.1X SSC, 0.5% SDS at Room Temperature for 15 minutes.

Low Stringency Hybridization:

6X SSC

0.5% SDS

100 ug/ml denatured salmon sperm DNA

50% formamide

Incubate overnight with gentle rocking at 37°C overnight.

Low Stringency Wash:

1 wash in 0.1X SSC, 0.5% SDS at Room Temperature for 15 minutes.

Moderately stringent conditions may be obtained by varying the temperature at which the hybridization reaction occurs and/or the wash conditions as set forth above.

Thus, as used herein, a sequence (A) is said to have "high homology" to another sequence (B) if sequence A hybridizes to sequence B under high stringency conditions (*i.e.*, high stringency hybridization and wash conditions as defined above), and if the polypeptide or polypeptide fragment encoded by sequence A has the same activity as the polypeptide encoded by B.

The present invention further provides a recombinant DNA fragment that includes a coding region for a transit peptide upstream of the coding region of an objective protein, such as mitochondrial superoxide dismutase. The expression of this recombinant DNA fragment enables to locate the objective protein in mitochondria. Thus, the present invention also provides a method for locating an objective protein in mitochondria which comprises expressing the recombinant DNA fragment containing a coding region for a transit peptide located upstream of the coding region of an objective protein in an appropriate recombinant host organism.

As mentioned above, the present invention discloses the nucleotide sequences of

active oxygen species-quenching factors, such as mitochondrial superoxide dismutase, cytoplasmic superoxide dismutase and catalase. These polynucleotides are provided for use as probes or primers for cloning the gene for active oxygen species-quenching factor(s) effective in other organisms capable of producing carotenoids, on the basis of the homology of the genes.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 shows activity staining for superoxide dismutase after native polyacrylamide gel electrophoresis by using cell-free extracts obtained from ATCC 96594 and its *SOD* mutants. Lane 1, *P. rhodozyma* ATCC 96594; Lane 2 *P. rhodozyma* ATCC 96594 :: pSOD/G717 (SOD1 disruptant); Lane 3 *P. rhodozyma* ATCC 96594 :: pSOD/G828 (SOD2 disruptant); Lane 4 *P. rhodozyma* ATCC 96594.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, one object of the present invention is to provide a novel process for producing carotenoids biologically. The novel process includes cultivating a recombinant organism whose gene for one or more active oxygen species-quenching factor(s) is substantially disrupted with the aid of a disruption cassette specific to the gene, and recovering carotenoids from the culture.

There is also provided a recombinant DNA sequence which contains an open reading frame coding for active oxygen species-quenching factor(s). Such a factor may be an enzyme, such as mitochondrial SOD or cytoplasmic SOD, or the recombinant DNA sequence may contain a partial fragment encoding a catalase gene. These sequences are useful for constructing the disruption cassette because they are able to recombine with the native gene(s) for the enzymes so as to specifically disrupt the gene(s).

The recombinant DNA sequence may be derived (*i.e.*, isolated) from an organism belonging to the kingdom of *Monera, Protista or Fungi*, more preferably to the genus *Erwinia, Rhodobacter, Myxococcus, Flavobacter, Paracoccus, Synechococcus, Synechocystis, Agrobacterium, Streptomyces, Haematococcus, Dunaliella, Phaffia, Xanthophyllomyces, Neurospora, Rhodotorula, Blakeslea,* or *Phycomyces*. A particularly preferred organism is *P. rhodozyma*.

The active oxygen species-quenching factor coded by the recombinant DNA sequence may be mitochondrial SOD, cytoplasmic SOD and/or catalase. A specific example of a recombinant DNA sequence is derived from a gene of *Phaffia rhodozyma*

and is selected from (i) a DNA sequence represented by SEQ ID NO: 1 or 2; (ii) those cDNAs identified by SEQ ID NO: 4 or 6; (iii) an isocoding or an allelic variant of the DNA sequence represented by SEQ ID NO: 1, 2, 4 or 6; and (iv) a derivative of a DNA sequence represented by SEQ ID NO: 1, 2, 4 or 6 with an addition, insertion, deletion and/or substitution of one or more nucleotide(s), and coding for a polypeptide having the enzyme activity. The recombinant DNA sequence may also be characterized as (a) coding for the enzyme having an amino acid sequence selected from the group of those described in SEQ ID NOs: 5 and 7, or (b) coding for a variant of the enzyme selected from (i) an allelic variant, and (ii) an enzyme having one or more amino acid addition, insertion, deletion and/or substitution and having the stated enzyme activity.

As used herein, an "allelic variant" means the variant that has at least one mutation in either one of two alleles in the diploid organism such as *Phaffia rhodozyma*, *Xanthophyllomyces dendrorhous* and the like. Both alleles of a given gene are concerned with the same trait or characteristic, but the product or function coded for by a particular allele differs, qualitatively and/or quantitatively, from that coded for by other alleles of that gene. Allelic variant can occur naturally or be generated artificially by means of chemical mutagenesis. A wild type allele is one which codes for a particular phenotypic characteristic found in the wild type strain of a given organism

As used herein, an "isocoding variant" means the variant in which the nucleotide sequence of a given gene differs from the sequence from the wild type gene although its translated product (i.e. amino acid sequence) is identical with that from the wild type protein. This is caused by degeneracy of genetic code and by the difference of codon usage which is not identical among various organisms.

As used herein, a "derivative of a DNA sequence" is a DNA sequence that encodes a polypeptide having the activity of the corresponding SEQ ID NO but which differs from that DNA sequence by from 1-20, preferably 1-10, such as 1-5 nucleotide additions, insertions, deletions, and/or substitutions.

The specified recombinant DNA sequence mentioned above may be derived from a gene of *Phaffia rhodozyma* and is selected from (i) a DNA sequence represented by SEQ ID NO: 3; (ii) cDNA identified by SEQ ID NO: 8; (iii) an isocoding or an allelic variant of the DNA sequence represented by SEQ ID NO: 3 or 8; and (iv) a derivative of a DNA sequence represented by SEQ ID NO: 3 or 8 with additions, insertions, deletions and/or substitutions of one or more nucleotides, and coding for a polypeptide having the enzyme activity. The recombinant DNA sequence may also be characterized by (a) coding for the

enzyme having a partial amino acid sequence selected from the group of those described in SEQ ID NO: 9, or (b) coding for a variant of the enzyme selected from (i) an allelic variant, and (ii) an enzyme having one or more amino acid additions, insertions, deletions and/or substitutions and having the stated enzyme activity. Such a recombinant DNA sequence may preferably be in the form of a vector.

The present invention also provides the use of the recombinant DNA sequence to transform a host organism to obtain an organism whose gene for at least one active oxygen species-quenching factor is substantially disrupted by introducing a disruption cassette specific to the gene. As used herein, a gene is "disrupted" or "substantially disrupted" if the activity of the polypeptide that it encodes is reduced relative to a non-disrupted gene. Preferably, the activity is reduced by 10%, preferably by at least 50% such as for example by at least 75%, more preferably by at least 90% to 100%.

A convenient form of the recombinant DNA sequence may be a vector. The recombinant organism obtained by use of the recombinant DNA is disrupted in its DNA sequence encoding mitochondrial SOD, cytoplasmic SOD, or catalase. The host organism transformed with the recombinant DNA is useful in improving the production process of carotenoids, in particular astaxanthin. Thus, the present invention also provides such a recombinant organism.

This biological production method of carotenoids may improve the productivity of carotenoids, in particular the productivity of astaxanthin. Thus, a method for producing a carotenoid is provided in which a recombinant microorganism as set forth above is cultivated under conditions conducive to the production of the carotenoid (see, e.g. the Examples) is one of the aspects of the present invention. This method may be applied to the biological production of astaxanthin.

Many researchers pointed out that active oxygen species might stimulate carotenoid production in known carotenogenic organisms. Carotenoid biosynthesis in cyst cells of *Haematococcus pluvialis* is enhanced by environmental oxidative stress (Kobayashi *et al.*, Appl. Env. Microbiol., 59, 867-873, 1993). Carotenoid biosynthesis might be induced by active oxygen species and the accumulated carotenoids might function as a protective agent against oxidative stress damage in *Dunaliella bardawil* (Shaish *et al.*, Planta, 190, 363-368, 1993). Although astaxanthin production in *P. rhodozyma* was studied *in vivo* under various cultivation conditions in which a generation of active oxygen species was altered, a correlation between active oxygen generated and carotenoids productivity was not clearly determined, probably because native active oxygen species-quenching factors were still present in such experiments and rescued the effects of active oxygen species on

the carotenoid production to some extent (Schroeder, W. A. *et al.*, J. Gen. Microbiol., 139, 907-912, 1993).

In this invention, to exclude the possibility that existence of native active oxygen species-quenching factor in *P. rhodozyma* can quench the positive effect by active oxygen on astaxanthin production, such native active oxygen species-quenching factors as SOD and catalase, were cloned from *P. rhodozyma* to disrupt their expression by constructing and introducing gene disruption plasmids. On the assumption that astaxanthin would play an antioxidant role in *P. rhodozyma*, inactivation of native active oxygen species-quenching factors may affect carotenoid production. This effect would probably occur because the relative increase in active oxygen species *in vivo*, due to absence of native active oxygen species-quenching factors, would stimulate a production of astaxanthin as an alternative agent for quenching active oxygen species.

Active oxygen species have toxicity to living cells because of the oxidative damage they cause to intercellular molecules, such as proteins or nucleic acids. Recent studies have revealed that aging is caused by oxidative damage by demonstrating a correlation between increased superoxide dismutase activity, increased life-span, and decreased oxidative damage in fruit flies and nematodes (Agarwal, S. *et al.*, Proc. Natl. Acad. Sci. U.S A., 91, 12332-12335, 1994, Larsen, P. L., Proc. Natl. Acad. Sci. U. S. A., 90, 8905-8909, 1993, Sohal, R. S. *et al.*, J. Biol. Chem., 270, 15671-15674, 1995). SOD and related antioxidant enzymes, and their genes, have been well studied in both prokaryotes and eukaryotes.

Yeast, such as *S. cerevisiae*, like most eukaryotes, contain Cu/ZnSOD (product of the *SOD1* gene) in the cytosol and MnSOD (product of the *SOD2* gene) in the mitochondria. These enzymes catalyze the disproportionating of $O_2\bullet$, producing O_2 and H_2O_2. Together with small molecular antioxidants, such as glutathione and ascorbate, and other antioxidant enzymes, such as catalases and peroxidases; and metal chelating proteins such as metallothionein, they allow aerobes to survive under O_2, presumably by minimizing oxidative damage. The importance of cytoplasmic SOD was demonstrated by the high sensitivity to dioxygen shown by *S. cerevisiae* and *Escherichia coli* devoid of *SOD*. In both organisms, the loss of SOD activity was associated with slow growth in aerobic conditions, with higher mutation rates and specific biosynthetic defects. (*sod*1$^-$ yeast requires lysine and methionine for aerobic growth, whereas *sod- E. coli* requires branched amino acids). In some cases, these effects are known to be due to the inhibitory effect of superoxide on iron sulfur cluster proteins (Gardner, P. R. *et al.*, J. Biol. Chem., 266, 19328-19333, .1991, Kuo, C. F., *et al.*, J. Biol. Chem., 262, 4724-4727, 1987).

Mutants of *sod2* of *S. cerevisiae* are little affected when grown in air with glucose as the carbon source. However, they are highly sensitive to hyperoxia and grow poorly in nomoxia, with carbon sources that require respiration for their metabolism.

Because genes coding for SOD and catalase have been cloned from other species, corresponding genes from *P. rhodozyma* can be cloned using the degenerate PCR method. At first, we cloned a partial gene fragment containing a portion of *SOD* gene and *CAT* gene by using the method set forth above. The degenerate PCR is a method to clone a gene of interest that displays high amino acid homology to the known enzyme of another species that has the same or similar function. The degenerate primer, which is used as a primer in degenerate PCR, was designed by reverse translation of the amino acid sequence of corresponding nucleotides ("degenerated"). In such a degenerate primer, a mixed primer which consists any of A, C, G or T, or a primer containing inosine at an ambiguity code is generally used. In this invention, such mixed primers were used as degenerate primers for cloning the genes mentioned above. As described hereinafter, varied PCR conditions were used depending on the gene primers used for cloning. In this invention, two species of *SOD* genes, with sequences different from each other, were cloned from the same PCR band via degenerate PCR and named *SOD1* and *SOD2*.

An entire gene containing its coding region and its intron, as well as its regulation region, such as a promoter or terminator, can be cloned from a chromosome by screening a genomic library with a partial DNA fragment obtained by degenerate PCR, as described above, as a probe after the probe is labeled. Generally, *E. coli* is used as a host strain and an *E. coli* vector, such as a λ phage vector or plasmid vector, such as a pUC vector, are often used in the construction of a library, following genetic manipulation such as sequencing, restriction digestion, ligation and the like. In this invention, an *EcoR*I genomic library of *P. rhodozyma* was constructed in the derivatives of λZAPII and λDASHII depending on an insert size. The insert size, in terms of the length of insert that must be cloned, was determined by Southern blot hybridization for each gene, prior to construction of a library. In this invention, the DNA was used as a probe was labeled with digoxigenin (DIG), a steroid hapten, instead of conventional ^{32}P label, following a protocol prepared by the supplier (Boehringer-Mannheim (Mannheim, Germany)). A genomic library constructed from the chromosome of *P. rhodozyma* was screened by using a DIG-labeled DNA fragment, which had a portion of a gene of interest, as a probe. Hybridized plaques were selected and used for further study. When λDASHII (insert size was from 9 kb to 23 kb), was used, the prepared λDNA was digested with *EcoR*I, followed by cloning of the *EcoR*I insert into a plasmid vector such as pUC19 or pBluescriptII SK+. When λZAPII was used in the construction of the genomic library,

an *in vivo* excision protocol was conveniently used for the succeeding step of the cloning onto the plasmid vector with a derivative of a single stranded M13 phage, Ex assist phage (Stratagene, La Jolla, USA). Plasmid DNA thus obtained was examined for sequencing. In this invention, *SOD1* and *SOD2* genes were obtained from the λZAPII library independently from each other and catalase (*CAT*) gene was cloned from λDASHII library.

In this invention, we used the automated fluorescent DNA sequencer, ALFred system (Pharmacia, Uppsala, Sweden) using an autocycle sequencing protocol in which the Taq DNA polymerase is commonly employed for sequencing.

In this invention, the inventors determined the genomic sequence, containing an open reading frame of *SOD1* gene or *SOD2* gene, as well as its promoter and terminator sequences. From sequence analysis, it was found that *SOD1* codes for a mitochondrial SOD, judged from the presence of a transit peptide at its amino terminal end. On the contrary, *SOD2* does not have such a transit peptide sequence suggesting that *SOD2* codes for a cytoplasmic SOD. The inventors also determined a partial genomic sequence of an open reading frame for the *CAT* gene.

A transit peptide is a signal sequence to transfer nucleic gene products that are encoded on a chromosome but whose translated proteins function in mitochondria, to the mitochondria, such as enzymes involved in the TCA cycle. To express some proteins in mitochondria, addition of a transit peptide at the protein's amino terminal end is useful.

In this invention, disruption plasmids for *SOD1*, *SOD2* and *CAT* genes were constructed by ligating partial fragments of the above genes, which do not contain either end of the genes, to drug resistant genes, thus creating a suicide vector that cannot be autonomously replicated in *P. rhodozyma* due to lack of an autonomous replication sequence. A drug resistant gene that encodes an enzyme that enables the host to survive in the presence of a toxic antibiotic is often used as a selectable marker. A G418 resistance gene harbored in pPR2T (Verdoes *et al.* (International patent publication, WO97/23633)) is an example of a drug resistance gene. Such a suicide vector cannot replicate by itself and can be present only in an integrated form on the chromosome of the host as a result of a single-crossing recombination using the homologous sequence between the vector and the chromosome. In the case of recombination with a gene of interest, its genetic sequence cannot be reconstituted on the chromosome of the host strain due to the lack of either end of the gene. As a consequence, the gene of interest could be disrupted in the recombinant strain thus obtained.

Another example for disruption plasmid is a double crossing over type of plasmid. This type of disruption plasmid contains two different partial fragments of the objective gene to be disrupted and a selective marker gene, such as a drug resistant gene, is inserted between the two fragments. After recombination between the chromosome of the recipient cell and donor plasmid DNA at the two homologous parts of the gene, replacement of the chromosome sequence with the donor DNA occurs and a selective marker gene is inserted into the objective gene that is to be disrupted. In general, a double crossing over type of plasmid has a lower frequency of recombination than single crossing over type of vector.

In this invention, the enzymes of interest were inactivated by disrupting the corresponding genes. The other way to evaluate the effect of gene product of interest is to decrease its expression by genetic engineering methods. For this purpose, some methods were used. One such method is the anti-sense method. The anti-sense method is used to decrease the expression of a gene of interest by introducing an artificial gene fragment whose sequence is complementary to that of the gene of interest. Such an anti-sense gene fragment forms a complex with the mature mRNA fragment of the objective gene *in vivo* and as a consequence, inhibits the efficient translation of the mRNA.

Another method is a mutation of the promoter region. In general, a gene consists of several parts that have different functions from each other. In eukaryotes, genes that encode corresponding proteins are transcribed to premature messenger RNA (pre-mRNA), which differs from the genes for ribosomal RNA (rRNA), small nuclear RNA (snRNA), and transfer RNA (tRNA). Although RNA polymerase II (PolII) plays a central role in this transcription event, PolII cannot solely start transcription without a *cis* element covering an upstream region containing a promoter and an upstream activation sequence (UAS), and a *trans*-acting protein factor. At first, a transcription initiation complex, which consists of several basic protein components, recognize the promoter sequence in the 5´-adjacent region of the gene to be expressed. In this event, some additional participants are required in the case of a gene which is expressed under some specific regulation, such as a heat shock response, or adaptation to a nutritional starvation, and so on. In such a case, a UAS is required to exist in the 5´-untranslated upstream region around the promoter sequence, and some positive or negative regulator proteins recognize and bind to the UAS. The binding strength of the transcription initiation complex to the promoter sequence is affected by the binding of a *trans*-acting factor around the promoter, thus enabling the regulation of transcription activity.

After the activation of a transcription initiation complex by phosphorylation, the transcription initiation complex initiates transcription from the transcription start site. Some parts of the transcription initiation complex detach as an elongation complex and progress from the promoter region to the 3´ direction of the gene (this step is called a promoter clearance event). The elongation complex continues transcription until it reaches a termination sequence that is located in the 3´-adjacent downstream region of the gene.

To decrease the expression of a gene of interest, mutation by conventional chemical mutagenesis or genetic site-directed mutagenesis in the promoter region of the objective gene containing a UAS sequence, as described above, is often used. Mutant strains in which the expression of an enzyme of interest might decrease can be obtained by transforming a host strain with recombinant DNA having such a mutated promoter region. As described above, such an attempt to decrease the expression of a gene, as well as gene disruption, are employed to determine the effect of a gene product on the phenomena of living organisms.

As a transformation method, LiCl method (Wery *et al.*, *Yeast*, 12 (7), 641-651, 1996) and electroporation method (Wery *et al.*, *Gene*, 184, 89-97, 1997) were applied to transform *P. rhodozyma*. However, the efficiency of transformation under these conditions seemed to be low. Thus, in this invention, the biolistic transformation method (Johnston *et al.*, *Methods in Molecular Biology*, 53; 147-153, 1996) was used for the transformation of *P. rhodozyma*. The biolistic method is a simple and reliable protocol in which donor DNA coated on a gold or tungsten particle is shot into the living cells directly with high-pressured helium gas. This transformation protocol was successfully applied to *Cryptococcus neoformans*, which is a basidiomycetous yeast, as well as *P. rhodozyma*, and was difficult to transform with conventional transformation methods (Toffaletti, *et al.*, *J. Bacteriol.*, 175 (5), 1405-1411, 1993). In this invention, this biolistic method was successfully used to transform *P. rhodozyma* cells.

The event of gene disruption can be confirmed by enzymatic characterization directly and by genetic analysis with PCR or Southern blot hybridization by using the chromosome obtained from transformants obtained as above. In this invention, the direct confirmation of SOD disruption was performed by activity staining. The characterization of catalase disruption was conducted by visual observation, such as the catalase test, which is often used in bacterial taxonomy.

Such a genetically engineered *P. rhodozyma* would be cultivated in an appropriate medium and evaluated for its productivity of astaxanthin.

The following materials and methods were employed in the Examples described below:

Strains

P. rhodozyma ATCC 96594 (re-deposited under the accession No. ATCC 74438 on April 8, 1998 pursuant to the Budapest Treaty).

E. coli DH5: F^-, ϕ80d, *lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *hsd* (r_K^-, m_K^+), *rec*A1, *end*A1, *deo*R, *thi*-1, *sup*E44, *gyr*A96, *rel*A1 (Toyobo, Osaka, Japan).

E. coli XL1-Blue MRF': Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac*[F' *pro*AB, $lacI^qZ\Delta M15$, Tn10 (tet^r)] (Stratagene, La Jolla, USA).

E. coli SOLR: e14$^-$(*mcr*A), Δ(*mcr*CB-*hsd*SMR-*mrr*)171, *sbc*C, recB, *rec*J, *umu*C :: Tn5(kan^r), *uvr*C, *lac*, *gyr*A96, *rel*A1, *thi*-1, *end*A1, λ^R, [F' *pro*AB, $lacI^qZ\Delta M15$] Su^- (nonsuppressing) (Stratagene).

E. coli XL1 MRA (P2): Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *lac* (P2 lysogen) (Stratagene).

E. coli TOP10: F^-, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80, Δ*lac*Z M15, Δ*lac*X74, *rec*A1, *deo*R, *ara*D139, (*ara-leu*)7697, *gal*U, *gal*K, *rps*L (Str^r), *end*A1, *nup*G (Invitrogen, Carlsbad, USA).

Vector

λZAPII (Stratagene)

λDASHII (Stratagene)

pBluescriptII SK+(Stratagene)

pCR2.1TOPO (Invitrogen)

pUC19 (Takara Shuzo, Otsu, Japan)

Media

The *P. rhodozyma* strain is maintained routinely on agar plates of YPD medium (DIFCO, Detroit, USA). The *E. coli* strain is maintained in LB medium (10 g Bacto-trypton, 5 g yeast extract (DIFCO) and 5 g NaCl per liter). NZY medium (5 g NaCl, 2 g $MgSO_4$-$7H_2O$, 5 g yeast extract (DIFCO), 10 g NZ amine type A (WAKO, Osaka, Japan) per liter is used for phage propagation in soft agar (0.7% agar (WAKO)). When agar medium was prepared, 1.5%(wt) of agar (WAKO) was supplemented.

Methods

A general method of molecular genetic techniques was used, according to the method in Molecular cloning: a Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory Press, 1989). Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Japan).

Isolation of a chromosomal DNA from *P. rhodozyma* was performed using QIAGEN Genomic Kit (QIAGEN, Hilden, Germany) following the protocol supplied by the manufacturer. Mini-prep isolation of plasmid DNA from transformed *E. coli* was performed with the Automatic DNA isolation system (PI-50, Kurabo, Co. Ltd., Osaka, Japan). Midi-prep isolation of plasmid DNA from an *E. coli* transformant was performed using QIAGEN column (QIAGEN). Isolation of λ DNA was performed with Wizard lambda preps DNA purification system (Promega, Madison, USA) following the protocol supplied by the manufacturer. DNA fragments were isolated and purified from agarose using QIAquick or QIAEX II (QIAGEN). Manipulation of λ phage derivatives was conducted using the protocol supplied by the manufacturer (Stratagene).

Isolation of total RNA from *P. rhodozyma* was performed with the phenol method by using Isogen (Nippon Gene, Toyama, Japan). mRNA was purified from total RNA thus obtained using mRNA separation kit (Clontech, Palo Alto, USA). cDNA was synthesized using CapFinder cDNA construction kit (Clontech).

In vitro packaging was performed using Gigapack III gold packaging extract (Stratagene).

Polymerase chain reaction (PCR) was performed with the Perkin Elmer model 2400 thermal cycler. Each of the PCR conditions used is described in the examples below. PCR primers were purchased from a commercial supplier or synthesized with a DNA synthesizer (model 392, Perkin Elmer, Japan, Urayasu, Japan).

Fluorescent DNA primers for DNA sequencing were purchased from Pharmacia. DNA sequencing was performed with the automated fluorescent DNA sequencer (ALFred, Pharmacia).

Competent cells of *E. coli* DH5α were purchased from Toyobo (Japan).

The apparatus and reagent for biolistic transformation of *P. rhodozyma* were purchased from Nippon Bio-Rad Laboratories (Tokyo, Japan).

The following examples are provided to further illustrate the process of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

Isolation of mRNA from *P. rhodozyma* and construction of a cDNA library

To construct cDNA library of *P. rhodozyma*, total RNA was isolated by the phenol extraction method right after cell disruption, and the mRNA from *P. rhodozyma* ATCC 96594 strain was purified using an mRNA separation kit (Clontech).

Cells of the ATCC96594 strain from 10 ml of a two-day-culture in YPD medium were harvested by centrifugation (1500 x g for 10 minutes) and washed once with extraction buffer (10 mM Na-citrate/HCl (pH 6.2) containing 0.7 M KCl). After suspending in 2.5 ml of extraction buffer, the cells were disrupted using a French press homogenizer (Ohtake Works Corp., Tokyo, Japan) at 1500 kgf/cm^2 and immediately mixed with two volumes of isogen (Nippon gene) according to the method specified by the manufacturer. 400 μg of total RNA was recovered.

This total RNA was purified using an mRNA separation kit (Clontech) according to the method specified by the manufacturer. Finally, 16 μg of mRNA from *P. rhodozyma* ATCC 96594 strain was obtained.

To obtain cDNA species from *P. rhodozyma*, CapFinder PCR cDNA construction kit (Clontech) was used according to the method specified by the manufacturer. One μg of purified mRNA was applied for a first strand synthesis followed by PCR amplification. After this amplification by PCR, 1 mg of cDNA pool was obtained.

Example 2

Cloning of two species of partial *SOD* gene from *P. rhodozyma*

To clone a partial *SOD* gene from *P. rhodozyma*, a degenerate PCR method was used. The nucleotide sequences of two mixed primers were designed and synthesized as shown in TABLE 1 based on the common sequence of known superoxide dismutase genes from other species.

TABLE 1: Sequence of primers used in the cloning of *SOD1* and *SOD2* genes

Sod1 ; AARCAYCAYCARACNTAYGTNAA (sense primer) (SEQ ID NO: 10)

Sod4 ; GCCCANCCNGANCCYTGNACNCC (antisense primer) (SEQ ID NO: 11)

(R=A or G; Y=C or T; N=A, C, G or T)

After a PCR reaction of 25 cycles at 94°C for 15 seconds, 46°C for 30 seconds and 72°C for 15 seconds, using ExTaq (Takara Shuzo) as a DNA polymerase and the cDNA pool obtained in Example 1 as a template, the reaction mixture was separated using agarose gel electrophoresis. A PCR band that had the desired length was recovered and purified by QIAquick (QIAGEN) according to the method of the manufacturer and then ligated to pCR2.1-TOPO (Invitrogen). After the transformation of competent *E. coli* TOP10, 6 white colonies were selected and plasmids were isolated with Automatic DNA isolation system (Kurabo PI-50). As a result of sequencing, it was found that two clones had different sequences from each other, and both amino acid sequences were independently similar to known *SOD* genes. These isolated cDNA clones were designated pSOD614 #2 and pSOD614 #3, and used for further study.

Example 3

Isolation of genomic DNA from *P. rhodozyma*

To isolate a genomic DNA from *P. rhodozyma*, a QIAGEN genomic kit was used according to the method specified by the manufacturer.

Initially, cells of *P. rhodozyma* ATCC 96594 strain from a 100 ml overnight culture in YPD medium were harvested by centrifugation (1500 x g for 10 minutes) and washed once with TE buffer (10 mM Tris/HCl (pH 8.0) containing 1 mM EDTA). After suspending in 8 ml of Y1 buffer of the QIAGEN genomic kit, lyticase (SIGMA) was added at the concentration of 2 mg/ml to disrupt cells by enzymatic degradation. The

reaction mixture was incubated for 90 minutes at 30°C and then continued on to the next extraction step. Finally, 20 μg of genomic DNA was obtained.

Example 4

Southern blot hybridization by using pSOD614 #2 and pSOD614 #3 as probes

Southern blot hybridization was performed to clone genomic fragments that contain *SOD* genes from *P. rhodozyma*. Two μg of genomic DNA were digested by *EcoR*I and subjected to agarose gel electrophoresis followed by acidic and alkaline treatment. The denatured DNA was transferred to a nylon membrane (Hybond N+, Amersham) using transblot (Joto Rika, Tokyo, Japan) for an hour. The DNA was transferred to a nylon membrane and fixed by heat treatment (80°C, 90 minutes). Probes were prepared by labeling the template DNAs (*EcoR*I- digested pSOD614 #2 and pSOD614 #3) with the DIG multipriming method (Boehringer Mannheim). Hybridization was performed using the method specified by the manufacturer. As a result, hybridized bands were visualized at a of 7.5 kilobases (kb) against the probe prepared from pSOD614 #2, and at a length of 8.0 kilobases (kb) against the probe prepared from pSOD614 #3.

Example 5

Cloning of genomic fragments containing *SOD* genes whose sequences were different from each other

Four μg of the genomic DNA were digested by *EcoR*I and subjected to agarose gel electrophoresis. Then, DNAs whose length are within the range from 7 to 9 kb were recovered by a conventional elution method using a dialysis membrane. The purified DNA was ligated to 1 μg of *EcoR*I-digested and CIAP (calf intestine alkaline phosphatase) -treated λZAPII (Stratagene) at 16°C overnight, and packaged with Gigapack III gold packaging extract (Stratagene). The *E. coli* XL1Blue MRF' strain was infected with the packaged extract and over-laid with NZY medium poured onto LB agar medium. About 6000 plaques were screened using *EcoR*I- digested pSOD614 #2 and pSOD614 #3 as probes. Six plaques were hybridized to the labeled pSOD614 #2 probe and two plaques were hybridized to the labeled pSOD614 #3 probe. Then, the hybridized plaques were subjected to an *in vivo* excision protocol according to the method specified by the manufacturer (Stratagene). As a result of PCR analysis using sod1 and sod4 primers, it was found that one plasmid isolated from the six pSOD614 #2-hybridized plaques had the same fragments as that of pSOD614 #2. This plasmid was named pSOD703. As a result

of PCR analysis using sod1 and sod4 primers, it was also found that two plasmids isolated from the two of pSOD614 #3-hybridized plaques had the same fragment as that of pSOD614 #3. One of the plasmids was named pSOD626 and used for further study.

Example 6

Sequence analysis of two species of MnSOD genes obtained from *P. rhodozyma*

A complete nucleotide sequence was determined by sequencing of pSOD703 and pSOD626 with a primer-walking procedure. The nucleotide sequence and the deduced amino acid sequence for the *SOD1* gene, which harbored on pSOD703, and for the *SOD2* gene, which is harbored on pSOD626, are provided as SEQ ID NOs: 1, NO: 2, NO: 5 and NO: 7 herein.

Both of the deduced amino acid sequences of the *SOD1* and *SOD2* genes were homologous to known MnSODs obtained from other species, and not to Cu/ZnSODs or FeSOD as a result of BLAST analysis (Altschul, S.F. *et al.*, *J. Mol. Biol.* 215, 403-410, 1990).

The *SOD1* gene had 7 introns and 8 exons. Its deduced open reading frame consisted of 223 amino acids. On the other hand, the *SOD2* gene had 10 introns and 11 exons, and its deduced open reading frame consisted of 199 amino acids. Most of the differences between the two isolated *SOD* genes occurred in an extended region of the *SOD*1 gene at its amino terminal end, whose sequence might act as a transit peptide to mitochondria

In fact, Schroeder *et al.* reported two species of SODs in *P. rhodozyma* which were detected as KCN- and H_2O_2- resistant SODs in the activity staining of native polyacrylamide gel electrophoresis (PAGE). They commented that two species of MnSOD were indicated as aggregates or isozymes, and they did not refer to the species' precise nature and their subcellular location. As described in the following section, it was clarified that two species of these KCN- and H_2O_2- resistant SODs (*i.e.* MnSOD) were products of the *SOD1* and *SOD2* genes. As described in the section of "Detailed Description of the Invention," most eukaryotes have different species of SODs located intracellularly (MnSOD in the mitochondrial fraction and Cu/ZnSOD in the cytoplasmic fraction). This is the first example in which two species of MnSODs have functioned in different subcellular locations.

Example 7

Construction of disruption plasmids for *SOD1* and *SOD2* genes

As described in the section "Detailed Description of the Invention," a plasmid harboring a drug resistant marker cassette was constructed by inserting G418 resistant structural gene between the promoter and terminator genes of glyceraldehyde-3-phosphate dehydrogenase (*GAP*) and ligating thus into *Kpn*I- and *Hind*III- digested pUC19. This plasmid was named pUC-G418 and used for further study. As gene fragments used for homologous recombination, partial fragments of the *SOD1* and *SOD2* genes were synthesized *in vitro* by the PCR method using PCR primers whose sequences were shown in TABLE 2.

Table 2

Sod14 : ; GGTACCTCCGATGATAGGAATGTGAG (sense primer) (SEQ ID NO: 12)

Sod15 : ; GAATTCAGTTCAACGGAGGAGGACAC (antisense primer) (SEQ ID NO: 13)

Sod47: ; GAATTCGGAGGAGGACACATCAACCG (sense primer) (SEQ ID NO: 14)

Sod48: ; GGTACCTGTACTGGAGGTAGAAAGCG (antisense primer) (SEQ ID NO: 15)

PCR conditions were as follows: 25 cycles of 94°C for 15 seconds, 50°C for 30 seconds, and 72°C for 15 minutes. As a template, 0.1 μg of genomic DNA obtained in Example 3 was used, and ExTaq was used as a DNA polymerase. A partial fragment of *SOD1* which could be obtained from PCR using sod14 and sod15 as primers, and a partial *SOD2* gene that could be obtained from PCR using sod47 and sod48 as primers, were amplified respectively. Amplified 0.65 kb fragments were recovered and cloned into pCR2.1-TOPO (Invitrogen) according to the protocol specified by the manufacturer. Six independent clones from white colonies of *E. coli* TOP10 transformants were selected, and plasmids were prepared from those transformants. As a result of restriction analysis and sequencing, one clone that had a partial *SOD1* gene was selected for a further study and named pSOD715. In a similar manner, one clone that had a partial SOD2 gene was selected and named pSOD826.

Then, pSOD715 and pSOD826 were digested by *Eco*RI and *Kpn*I, yielding 0.65kb fragments that were purified using QIAquick protocol and ligated to *Eco*RI- and *Kpn*I-

digested pUC19-G418. Six independent clones from ampicillin-resistant colonies of *E. coli* DH5α transformants were selected and plasmids were prepared from those transformants. As a result of restriction analysis and sequencing, one clone in which a partial *SOD1* was fused to the G418 resistant cassette was obtained and named pSOD/G717. In a similar manner, one clone in which a partial *SOD2* was fused to the G418 resistant cassette was obtained and named pSOD/G828.

Example 8

Transformation of *P. rhodozyma* ATCC 96594 using the biolistic method

Transformation protocols were followed according Methods in Molecular Biology (Johnston *et al.*, 53; 147-153, 1996). As a host strain, *P. rhodozyma* ATCC 96594 was cultured in YPD medium to stationary phase. After centrifugation of the broth, cells were concentrated 10-fold with sterilized water. 200 μl of cell suspension were spread on YPD medium containing 100 μg of geneticin, and 0.75 M of mannitol and sorbitol. Five micrograms of the circular DNA of pSOD/G717 and pSOD/G828 were coated on 1.5 mg of 0.9 μm gold particles, and used as donor DNA for biolistic transformation. Hundreds of geneticin-resistant clones were obtained after one week of incubation at 20°C. Four of those transformants were selected and chromosomes were prepared from them. One of the transformants was confirmed to have this disrupted structure of chromosomal *SOD1* or *SOD2* genes by PCR and Southern blot hybridization analyses, and was used for further study.

Example 9

Activity staining of native PAGE using crude extracts obtained from candidates for SOD1 and SOD2 disruptants

ATCC 96594 strain and candidates obtained from the biolistic transformation of ATCC 96594 were cultivated in YPD medium for two days and harvested by centrifugation for 10 minutes by 3000 x g at 4°C. After washing with Tris-HCl buffer (10mM/pH 8.0), cells were concentrated 10-fold with the same buffer. Cells were disrupted with a French press homogenizer (Ohtake Works) at 1500 kg/cm^2 and a crude extract was prepared after microcentrifugation at 15000 rpm (TOMY, MRX150) of homogenized fraction.

The protein concentration of the crude extract, thus prepared, was determined with BCA protein assay reagent manufactured by PIERCE (Rockford, U.S.A.). A

volume of crude extract corresponding to 300 μg of protein was subjected to native PAGE according to the method described by Schroeder W. A. *et al.* (*J. Gen. Microbiol.*, 139, 907-912, 1993). Activity staining was conducted according to the method of Flohé *et al.* (*Methods in Enzymology*, 105, 93-104, 1984).

The results of the activity staining are depicted in FIG. 1. In the extract of parental strain, ATCC 96594, two bands were visualized as transparent bands that have SOD activity in the dark background. On the contrary, the ATCC 96594 :: pSOD/G717 strain, in which the *SOD1* gene was disrupted, lacked an activity band with high mobility in native PAGE and ATCC 96594 :: pSOD/G828 strain, in which the *SOD2* gene was disrupted, lacked an activity band with low mobility in native PAGE. From this result, it was found that two species of MnSOD that were present in the crude extract of *P. rhodozyma* were the products of *SOD1* and *SOD2* genes, and the SOD species with high mobility and with low mobility in native PAGE corresponded to the *SOD1* and *SOD2* gene product, respectively.

<u>Example 10</u>

Cloning of the partial catalase *(CAT)* gene from *P. rhodozyma*

To clone a partial *CAT* gene from *P. rhodozyma*, a degenerate PCR method was exploited. Two mixed primers were designed and synthesized as shown in TABLE 3 with their nucleotide sequences based on the common sequence of known catalase genes from other species.

Table 3: Sequence of primers used in the cloning of the *CAT* genes

Cat2 : MGNTTYTCNACNGTNGGNGGNGA (sense primer) (SEQ ID NO: 16)

Cat5 : CKRTGNCKYTGNGTRTCNGGRTA (antisense primer) (SEQ ID NO: 17)
(M=A or C; N=A, C, G or T; Y=C or T; K=G or T; R=A or G)

After the PCR reaction of 25 cycles of 94°C for 15 seconds, 45°C for 30 seconds and 72°C for 15 seconds, using ExTaq (Takara Shuzo) as a DNA polymerase and genomic DNA obtained in Example 3 as a template, the reaction mixture was applied to agarose gel electrophoresis. A PCR band of 1.0 kb length was recovered and purified by QIAquick (QIAGEN) according to the method of the manufacturer and then ligated to pCR2.1-TOPO (Invitrogen). After the transformation of competent *E. coli* TOP10, 6 white colonies were selected and plasmids were isolated with Automatic DNA isolation system. As a result of sequencing, it was found that two clones had sequence whose

deduced amino acid sequences were similar to known *CAT* genes. One of these isolated DNA clones was designated as pCAT702 and used for further study.

Example 11

Cloning of genomic fragments containing the *CAT* gene

In a similar manner to Example 4, a Southern blot hybridization study was performed using pCAT702 as a probe. As a result, a hybridized band with a size from 9 kb to 23 kb was visualized. Next, 4 μg of the genomic DNA was digested by *EcoR*I and subjected to agarose gel electrophoresis. Then, DNAs whose length is within the range from 9 to 23 kb were recovered by a conventional elution method using a dialysis membrane. The purified DNA was ligated to 1 μg of *EcoR*I-digested and CIAP (calf intestine alkaline phosphatase) -treated λDASHII (Stratagene) at 16°C overnight, and packaged by Gigapack III gold packaging extract (Stratagene). The packaged extract was infected to *E. coli* XL1Blue MRA(P2) strain and over-laid with NZY medium poured onto LB agar medium. About 8000 plaques were screened using *EcoR*I- digested pCAT702 as a probe. Six plaques were hybridized to the labeled pCAT702 probe. λDNA was prepared from each λ clone and it was found that 4 of 6 clones contained same fragment to the insert of pCAT702 as a result of PCR using Cat2 and Cat5 primers, and sequencing analysis. A partial nucleotide sequence and its deduced amino acid sequence for *CAT* gene are listed as SEQ ID NO: 3 and SEQ ID NO: 9 in the sequence listing section.

Example 12

Construction of disruption plasmid for the *CAT* gene

In a manner similar to Example 7, a disruption plasmid for the *CAT* gene was constructed. At first, *Sac*I linker was inserted at a *Hind*III site of pUC19-G418, in which the terminator region of a G418-resistant cassette was located, and as a result of restriction analysis, pUC19-G418Sa, which had a *Sac*I site at the end of the G418-resistant cassette was obtained. Then, a *Kpn*I- and *Sac*I- fragment derived from pUC19-G419Sa was ligated to *Kpn*I- and *Sac*I- digested pCAT702 and yielded pCAT/G706, in which a partial genomic *CAT* gene was fused to G418-resistant cassette.

Example 13

Transformation of *P. rhodozyma* ATCC 96594 using pCAT/G706 as a donor

In a similar manner as set forth in Example 8, *P. rhodozyma* ATCC 96594 was transformed with a *CAT*-disruption plasmid, pCAT/G706. Hundreds of geneticin-resistant clones were yielded after one week of incubation at 20°C. Four of those transformants were selected and the chromosomes were prepared from them. One of the transformants was confirmed to have a disrupted structure of the chromosomal *CAT* gene by PCR and Southern blot hybridization analyses, and was used for further study.

Subsequently, two candidates for *CAT* disruptants were characterized with the catalase test, which was often used in bacterial taxonomic study. One loopful of *P. rhodozyma* cells was soaked in 3% H_2O_2 solution and the occurrence of dioxygen gas was observed. Although the immediate occurrence of O_2 foam was confirmed when ATCC 96594 cells were applied to this catalase test, O_2 foam occurred after a long lag when two ATCC 96594 :: pCAT/G706 mutants were soaked in H_2O_2 solution. From this result, the disruption of the *CAT* gene was suggested, but the remaining weak activity indicated the presence of another player who catalyzes the disappearance of H_2O_2 such as peroxidase in *P. rhodozyma*.

Example 14

Evaluation of *SOD1*, *SOD2* and *CAT* disruptants derived from *P. rhodozyma* for their astaxanthin production

The effect of gene disruption of *SOD*1, *SOD*2 and *CAT* gene on astaxanthin production was evaluated by cultivation in YPD medium with shaking flasks. Cells which grew on YPD agar were suspended in YPD medium and a portion of cell suspension was inoculated to 50 ml of YPD medium in 500 ml baffled flask. Cells were grown with 200 rpm at 20°C for 84 hours. At an appropriate interval, 3 ml of broth was withdrawn and was analyzed for cell yield, consumption of glucose and astaxanthin content.

Cell yield was measured as optical density at 660 nm and as dry cell weight by weighing cells after filtration through 0.45 μm cellulose acetate plus nitrocellulose membrane (HAWP04700, Millipore, Bedford, U.S.A.) and heating at 80°C overnight. Astaxanthin content of *P. rhodozyma* was measured with HPLC method after extraction of carotenoids from cells of *P. rhodozyma* by disruption with glass beads. After extraction, 5ml of a mixture containing acetone/BHT/water containing appropriate concentration of

bixin as an internal standard was added. Supernatant was analyzed for astaxanthin content with the following HPLC system.

HPLC column; YMC-Pak ODS-A (6 mm, 150 mm)

Temperature; room temperature

Eluent; acetonitrile / methanol / isopropanol (85 / 10 / 5)

Injection volume; 10 μl

Flow Rate; 2.0 ml/minute

Detection; UV at 471 nm

Results obtained from 84 hour-culture are summarized in TABLE 4.

Table 4: The effect of *SOD* and *CAT* mutation on the productivity of total carotenoids and astaxanthin by *P. rhodozyma*

Strain	Total carotenoids (mg/g-dry-cell)	astaxanthin (mg/g-dry cell)
ATCC 96594	0.169	0.111
Δ*SOD1*	0.259	0.146
Δ*SOD2*	0.202	0.129
Δ*CAT*	0.229	0.144

SOD1 and *SOD2* disruptants showed elevated level of productivity for total carotenoids as well as astaxanthin compared to their host strain, ATCC 96594. Especially, *SOD1* disruptant showed significant increase of carotenoids and astaxanthin production by 53.3 % and 31.5 %, respectively.

SOD1 seemed to be a mitochondrial enzyme judged from deduced transit peptide sequence at its amino terminal end and might act to scavenge superoxide radical, a kind of active oxygen species occurred in the respiratory chain at mitochondria. These data suggested that astaxanthin production was stimulated by a generation of intracellular active oxygen to compensate the lack of native player of active oxygen species-quenching factor, SOD1.

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the following claims.